Lysis of cholinergic synaptosomes by an antiserum to choline acetyltransferase

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1. INTRODUCTION

Synaptosomes have proved most useful in investigating the biochemistry of the synapse [1]. A major limitation in the use of synaptosomes from mammalian brain is the heterogeneity of the preparations with respect to transmitter type. An ability to discriminate between sub-populations of nerve-terminals would allow these sub-populations to be counted, and even separated in a manner that has been found possible for subclasses of lymphocytes [2]. Elimination of a sub-population would allow greater certainty in attributing specific properties to a nerve-ending type (e.g., in studying pre-synaptic receptors [3]).

Antisera to synaptosomes have been described which affect synaptosomal properties [4–8]; however, only a few studies have used antisera seemingly capable of selectively affecting sub-populations of synaptosomes [9,10].

Here, an antiserum to a purified choline acetyltransferase preparation, acting with complement, appeared to lyse cholinergic synaptosomes, while having no action on synaptosomes capable of taking up γ-amino-butyric acid (GABA).

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Abbreviations: ChAT, choline acetyltransferase (EC 2.3.1.6); DABA, 2,4-di-amino-butyric acid; GABA, γ-amino-butyric acid; LDH, lactate dehydrogenase (EC 1.1.1.27)

2. METHODS

An antiserum was prepared from the partially purified bovine enzyme (Sigma) by injection of an oil-in-water emulsion prepared using Freund's complete adjuvant. The emulsion, containing ~ 1 mg protein, was injected intramuscularly into a Californian rabbit; 51 days after the initial immunization a further 100 μg protein was injected intradermally into a site on the back. Blood samples were taken before and at several intervals during the course of immunization.

The presence in the serum of antibodies to the choline acetyltransferase (ChAT) preparation, was tested by double immunodiffusion [11]. Dilutions of the antiserum were tested against a solution of the antigen in phosphate-buffered saline.

Direct immunoprecipitation of ChAT activity from a rat brain soluble supernatant fraction (11 000 × *g*/40 min, [12]) was achieved using dilutions of the antiserum and Pansorbin (Gibco Labs.). Briefly, in 1.0 ml a total vol. dilutions of antiserum were incubated at room temperature for 1 h with the rat brain supernatant showing ChAT activity. An aliquot (50 μl) of resuspended *Staphylococcus* A cells (Pansorbin) was introduced, and the incubation allowed to continue at room temperature for a further 30 min. *Staphylococcus* A cells were removed from the suspension by centrifugation in a bench ultracentrifuge for 5 min. The enzyme was assayed in the supernatant and the resuspended pellet using 0.4 mM [1-^{14}C]acetyl coenzyme A (4 mCi/mmol, Amersham International) by the method in [13].

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The sodium-dependent uptake of [^{3}H]choline (15 Ci/mmol, Amersham International) into rat cerebrocortical synaptosomes [14] was measured at 0.0625–2 μM choline as in [16], using smaller volumes (500 μl) in Eppendorf tubes, in triplicate. In some experiments the action of 1 μM hemicholinium on choline uptake was tested. This was present during the 5 min preincubation of the synaptosomes before the choline was added. Sodium-dependent uptake 4-amino-*n*-[2,3-^{3}H]butyric acid, at 0.125–4 μM, into synaptosomes was measured by the same method. In some experiments synaptosomes were preincubated with Krebs medium containing 100 μM 2,4-diamino-butyric acid (DABA) prior to the assay of GABA uptake. Uptake of GABA was also assayed in synaptosomes that had been lysed in 8 mM Tris–HCl (pH 8.1) (1 mg synaptosomal protein/10 ml) for 30 min at 0–4°C. Synaptosomes were pelleted at 3000×*g* and re-suspended in 0.32 M sucrose at 2 mg protein/ml. Eadie-Hofstee plots of V against V/S, were constructed to determine V_{max} and K_m-values. Straight lines were fitted to the data by linear regression.

Lactate dehydrogenase (LDH) activity was measured as in [16].

The actions of the antisera were tested by incubating cortical synaptosomes (400 μg protein/ml) in 10 ml sodium-free Krebs' Ringer phosphate which contained either antiserum (0.2 or 2.0 ml) plus 1 ml of guinea pig complement (Gibco Labs), or the antiserum alone, for 30 min at 37°C. After this incubation, the synaptosomes were deposited by centrifugation at 3000×*g*. The supernatant was retained for LDH assay, and the pellets were first surface-washed with sodium-free Krebs' Ringer before being resuspended in 0.32 M sucrose (2 mg synaptosomal protein/ml) for the assay of choline or GABA uptake, where 50 μl of this suspension was added to 500 μl final incubation vol. LDH activity in the pellets was measured after lysis produced by the addition of 8 mM Tris–HCl (pH 8.1) and freezing and thawing, twice.

3. RESULTS AND DISCUSSION

A dilution of 1:16 of the antiserum with phosphate-buffered saline produced a precipitate in the double immunodiffusion test against the partially purified ChAT preparation. The more sensitive method of direct immunoprecipitation caused 100% precipitation of ChAT activity without loss of activity (769 ± 96 pmol . 15 min^{-1} . mg rat brain protein^{-1}, 6 separate prep) at a dilution of antiserum of 1:5. *Staphylococcus* A cells incubated in the absence of antiserum were capable, however, of precipitating 20% of the enzyme activity.

The results of table 1 show that the antiserum caused total suppression of the sodium-dependent uptake of choline at a dilution of 1:5 in the incubation medium, provided complement was added. Sodium-independent uptake was unaffected, accounting for 25% of the total uptake at 0.5 μM choline (not shown). A dilution of antiserum of 1:50 caused a halving of the V_{max} without altering the K_m, showing a dose-dependent relationship. Antiserum alone at these dilutions caused an increase in K_m, the 1:5 dilution producing a 2-fold change.

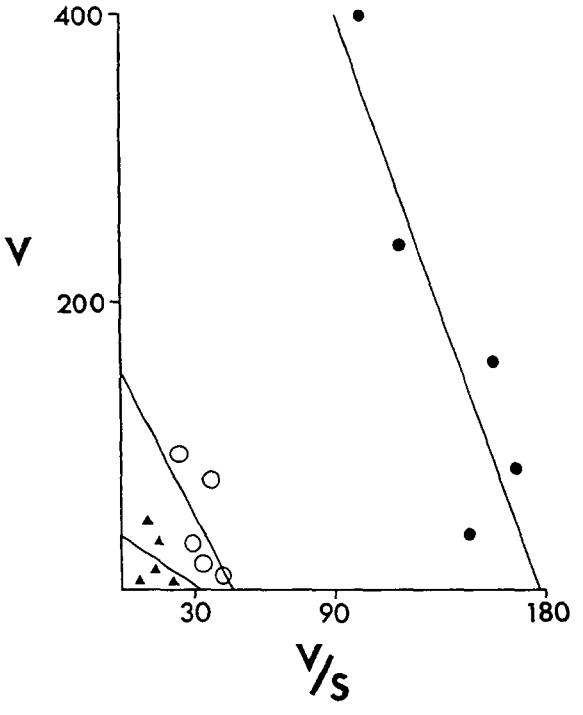

Fig.1. Typical Eadie – Hofstee plots of sodium-dependent GABA uptake into control (•—•) and lysed (○—○) synaptosomes, and synaptosomes incubated with 100 μM DABA (▲—▲). Data shown are the mean of triplicate determinations at each GABA concentration. Lines through the points were determined using linear regression analysis.

Table 1

Effects of partially purified choline acetyltransferase antiserum on cerebrocortical synaptosomes prepared from rat

Treatment	Sodium-dependent choline uptake		Sodium-dependent GABA uptake		Lactate dehydrogenase activity (% release)
	V_{max}	K_m	V_{max}	K_m	
Krebs medium	55 ± 5	0.54 ± 0.05	780 ± 69	4.9 ± 0.5	4– 6%
Pre-immune antiserum (1:5) + complement	59 ± 7	0.52 ± 0.06	760 ± 92	5.1 ± 0.6	5– 8%
Anti-serum (1:50) + complement	27 ± 5	0.55 ± 0.04	Not est.	Not est.	16–20%
Antiserum (1:5) + complement	Not meas.	Not meas.	680 ± 80	5.4 ± 0.7	30–34%
Antiserum (1:50)	56 ± 4	0.58 ± 0.05	Not est.	Not est.	5– 8%
Antiserum (1:5)	58 ± 6	1.2 ± 0.09	Not est.	Not est.	5– 7%
Complement	52 ± 5	0.53 ± 0.05	772 ± 80	5.2 ± 0.5	4– 8%

Values given for uptake are mean ± SD from 7 separate synaptosome preparations. Total lactate dehydrogenase activity in synaptosomes was 1.28 ± 0.11 μmol . mg synaptosomal protein^{-1}. min^{-1}. V_{max} is given in units of pmol . mg synaptosomal protein^{-1} . 2 min^{-1} . K_m is given in units of μM.

This effect was similar to the action of 0.1 μM hemicholinium, which is known to block uptake in a competitive manner (not shown). Pre-immune serum and complement, and complement alone, however, were without effect.

The sodium-dependent uptake of GABA was not changed by the antiserum, with respect to either V_{max} or K_m, though DABA (100 μM) could substantially reduce its uptake (92% reduction of control at 1 μM GABA, fig.1). Sodium-independent uptake was similarly unaffected by the antiserum, accounting for ~ 10% of the uptake at 1 μM GABA.

Measurement of LDH showed that this soluble enzyme was released into the incubation medium to the extent of 30–34% by the antiserum at the 1:5 dilution, and by 16–20% at the 1:50 dilution, provided complement was present. Neither complement alone, nor antiserum alone caused any release of LDH control levels.

The results indicate that the antiserum contains antibodies able to lyse ~ 1/3rd of cortical synaptosomes, whilst totally preventing sodium-dependent choline uptake. The lack of action on GABA uptake indicates a degree of specificity in the lysis, and suggests it may be confined to cholinergic terminals. GABA uptake was reduced to low levels by hypotonic lysis. Uptake no longer conformed to Michaelis–Menten kinetics (fig.1; $r = -0.51$, $y = 130–2.57x$).

If the antiserum and complement has caused

similar membrane lesions on GABA-terminals, GABA uptake should have been affected.

The immunoprecipitation tests are consistent with the presence of antibodies to ChAT. If interaction between ChAT antibodies and complement is the mechanism leading to synaptosomal lysis, then ChAT is presumably present and accessible in the outer surface membrane of cholinergic synaptosomes. Should ChAT antibodies and complement gain acces to the inner surface of the membrane, then lysis could result, but this seems a less tenable explanation.

In [10,17] similar complement-mediated lysis (i.e., LDH release) of guinea-pig cortical synaptosomes was reported in the presence of a polyclonal antiserum to the nerve-terminals (T-sacs) of *Torpedo* electric organ, but have not identified the specific antigens responsible. In [8] antisera to whole synaptosomes in the presence of complement cause increased permeability to K^+ and Ca^{2+} but not to proteins such as LDH. The differences in these results may be explained by varying treatment procedures, and by the nature and distribution of the antigens involved.

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